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Inhibition of *Clostridium botulinum* 62A by Saturated n-Aliphatic Acids, n-Alkyl Formates, Acetates, Propionates and Butyrates

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ABSTRACT

Saturated aliphatic acids (C_1 to C_{20}) and n-alkyl formate, acetate, propionate and butyrate esters (C_1 to C_{20}) were evaluated to determine the minimum inhibitory concentrations (MIC) necessary to inhibit the growth of *Clostridium botulinum* 62A in a bacteriological medium. The C_1 to C_7 and C_{16} to C_{20} acids and esters were relatively inactive (MIC > 200 μg/ml). The C_8 to C_{15} acids exhibited some antibotulinal acitivity (MIC = 100 μg/ml), whereas C_8 to C_{15} esters were substantially more inhibitory. The n-alkyl formates, acetates and propionates having C_{13} to C_{14} groups gave the highest inhibition, with MIC's of 3.1, 6.3 and 6.3 μg/ml, respectively. The maximum inhibition for the butyrates (MIC = 12.4 μg/ml) was associated with C_{11} to C_{12} alkyl groups. A mathematical relationship between activity and alkyl group chain length was derived.

Fatty acids and their esters are well-known for their antimicrobial activity, and the relationship between activity and molecular structure has been reviewed (*8,10*). Kabara (*8*) concluded that the greatest antibacterial activity is exhibited by $C_{16:1}$ mono- and $C_{18:2}$ polyunsaturated acids, and that within a homologous series, the *cis*-form is more active than the corresponding *trans*-form. Aliphatic acids show maximum activity at a chain length of C_{10} to C_{12}. It has also been noted that long-chain fatty acids are more effective against gram-positive bacteria, whereas gram-negative are more susceptible to short-chain fatty acids (*8,10,13*).

The monoesters of polyhydric alcohols are typically more active than the corresponding polyesters, with monolaurin-glyceride being the most active ester tested to date (*8*). Reviewing the antifungal activity of fatty acids and their derivatives, Gershon and Shanks (*4*) concluded that the activity of these compounds is dependent on the chain length of the molecule and the pH of the medium. Fatty acids are less active at elevated pH values; however, pH changes have little or no effect on the activity of the esters. Likewise, the presence of materials such as beef serum, proteins, starch, charcoal, cholesterol, lecithin and calcium or magnesium ions reduce the activity of fatty acids but not their esters.

Several investigators have concluded (*8,10,11,13*) that the importance of fatty acids and their esters as food preservatives has been largely overlooked. Because there have been a limited number of studies on the effects of fatty acids and their esters on *Clostridium botulinum (5,6)*, the objective of the present study was to systematically screen saturated aliphatic acids (C_1 to C_{21}), and their corresponding alkyl formates, acetates, propionates and butyrates, for their effects on this microorganism. In part, this study was undertaken to determine if these compounds have sufficient antibotulinal activity to act as potential replacements for sodium nitrite.

MATERIALS AND METHODS

Chemicals

Formic acid (98%) was obtained from Baker & Adamson General Chemical Co.[1]; glacial acetic acid, propionic and butyric acids were obtained from J. T. Baker Chemical Co.; all the alcohols, except 1-heptadecanol, were obtained from Aldrich Chemical Co.; and methyl, ethyl, propyl and butyl acetates propionates, butyrates, and 1-heptadecanol were obtained from Eastman Organic Chemicals. All the formates and the remaining higher acetates, propionates and butyrates (Table 1) were prepared in this laboratory.

Preparation of esters

Amyl to eicosanyl formates were prepared using the procedure of Dymicky (*2*). All synthesized alkyl acetates, propionates and butyrates were prepared using the procedure outlined below for n-amyl acetate. n-Amyl alcohol (0.1 mole) was added to a three-neck reaction flask, which was equipped with a condenser, stirrer and addition funnel, and placed in a 40°C silicon bath. Acetyl chloride (0.1 mole) was then added dropwise over a 10-min period, and allowed to react for an additional 15 min. n-Hexane (150 ml) was added, followed 5 min later by triethylamine (0.1 mole), and allowed to react for 30 min. Precipitated triethylamine hydrochloride was filtered off, washed with 50 ml of n-hexane, and the filtrates combined and distilled. The product boiling at 147 to 149°C was collected (yield = 92 to 95%).

The identity of the synthesized esters was confirmed by determining boiling points, densities, indices of refraction, molecular refraction (*9*),

[1]*Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.*

TABLE 1. *Inhibition of* C. botulinum *by n-aliphatic acids and n-alkyl formates, acetates, propionates and butyrates.*

R	Minimum inhibitory concentration (MIC, μg/ml) of				
	RCOOH	HCOOR	CH_3COOR	CH_3CH_2COOR	$CH_3CH_2CH_2COOR$
H	>200	—	—	—	—
Methyl	>200	≥200	≥200	≥200	≥200
Ethyl	>200	≥200	≥200	≥200	≥200
Propyl	>200	≥200	≥200	≥200	≥200
Butyl	>200	≥200	200	200	≥200
Amyl	>200	≥200	200	200	200
Hexyl	>200	200	200	200	200
Heptyl	200	100	200	200	200
Octyl	100	50	100	100	100
Nonyl	100	25	25	50	50
Decyl	100	25	25	50	25
Undecyl	100	12.5	25	50	12.5
Dodecyl	100	6.3	25	25	12.5
Tridecyl	100	3.1	6.3	6.3	25
Tetradecyl	100	3.1	6.3	6.3	25
Pentadecyl	100	12.5	—	—	—
Hexadecyl	200	25	12.5	≥25	50
Heptadecyl	>200	50	50	100	100
Octadecyl	>200	≥200	≥200	≥200	≥200
Nonadecyl	>200	≥200	≥200	≥200	≥200
Eicosanyl	>200	≥200	≥200	≥200	≥200

and IR spectra, and comparing against published values. The IR spectra (film 0.025 mm) matched with the authentic spectra (*2,12,14*). The purity of lower esters esterified with C_1 to C_{11} saturated n-aliphatic alcohols was further established by gas-liquid chromatography (model 4708, Nuclear-Chicago, Inc.) using the procedure of Byars and Jordan (*1*).

Activity against C. botulinum *type A*

Stock solutions. Stock solutions of the test compounds were prepared by dissolving 10 mg of a compound in 1.0 ml of 95% ethanol.

Medium. Assay medium consisted of yeast extract, 5 g; tryptone, 5 g; nutrient broth (Difco), 2.7 g; K_2HPO_4, 1.2 g; glucose, 2 g; sodium thioglycolate, 0.5 g; and deionized water, 1000 ml. The meidum (pH 7.25) was dispensed in 5-ml portions into test tubes (15 × 125 mm), and autoclaved for 15 min at 15 psi.

Activity determination. Minimum inhibitory concentrations (MIC) of the test compounds were determined using the procedure described by Huhtanen (*7*). Duplicate tubes of sterile, deoxygenated medium were supplemented with the appropriate volumes of test compound stock solution to achieve final concentrations of 200, 100, 50, 25, 12.5, 6.1, 3.1 and 0 μg/ml. A 24-h vegetative culture was diluted in sterile deoxygenated medium, and subsequently used to inoculate the supplemented tubes. Each tube received an inoculum of 10 μl, resulting in an inoculum level of approx. 300 cells/ml. All tubes were incubated anaerobically for 24 h at 30°C in a controlled atmosphere incubator. The samples were then inspected visually for turbidity, and the MIC (μg/ml) estimated. Extended incubation (≥72 h) had little effect on the observed results.

RESULTS AND DISCUSSION

The effects of n-aliphatic acids and n-alkyl formates, acetates, propionates and butyrates on *C. botulinum* are summarized in Table 1. The shorter chain (C_1 to C_7) fatty acids (column 1, Table 1) had relatively little activity (MIC > 200 μg/ml) as compared to sodium nitrite (MIC ~ 80 μg/ml). The activity of the C_8 to C_{15} fatty acids was greater (MIC ~ 100 μg/ml), whereas the longer chain ($\geq C_{17}$) acids had less activity (MIC > 200 μg/ml). This pattern of inhibition was similar to that noted previously for *Clostridium perfringens* (*8*).

The activity of esters formed by reacting various chain length n-aliphatic alcohols with formic, acetic, propionic or butyric acids displayed a pattern similar to that noted for the aliphatic acids (Table 1, column 2-5). The shorter (C_1 to C_6) and longer ($\geq C_{18}$) chain esters had little activity (MIC > 200 μg/ml), whereas significant activity was observed with the medium chain length (C_7 to C_{17}) esters. Maximum inhibitory acitivty against *C. botulinum* was observed with the C_{13} and C_{14} formates (MIC = 3.1 μg/ml), acetates, propionates (MIC = 6.3 μg/ml), and the C_{11} and C_{12} butyrates (MIC = 12.5 μg/ml). It was apparent that the medium chain length alkyl esters had considerably more activity than the corresponding aliphatic acids.

The relationship between activity (ln MIC) and chain length (R) was further analyzed by polynomial regression analysis. The cubic equations depicted below express the significant ($P < 0.05$) relationships associated with the formates (F), acetates (A), propionates (P) and butyrates (B).

$$\ln MIC = 2.06 + 1.84\ R - 0.28\ R + 0.01\ R^3 \quad (F)$$
$$\ln MIC = 10.84 - 1.18\ R + 0.05\ R^2 + 0.01\ R^3 \quad (A)$$
$$\ln MIC = 0.87 + 2.05\ R - 0.25\ R^2 + 0.01\ R^3 \quad (P)$$
$$\ln MIC = -1.98 + 3.08\ R - 0.38\ R^2 + 0.01\ R^3 \quad (B)$$

This relationship for the formates is also depicted graphically in Fig. 1, and compared against the experimental data.

It has been proposed that the antimicrobial activity of n-aliphatic acid is due to an inhibition of membrane transport resulting from a disruption of the proton motive force (*3*). Whether or not the antibotulinal activity of n-alkyl esters involves a similar mechanism awaits future research. However, the compounds do have significant activity against *C. botulinum* and warrant further investigation as potential antimicrobial agents.

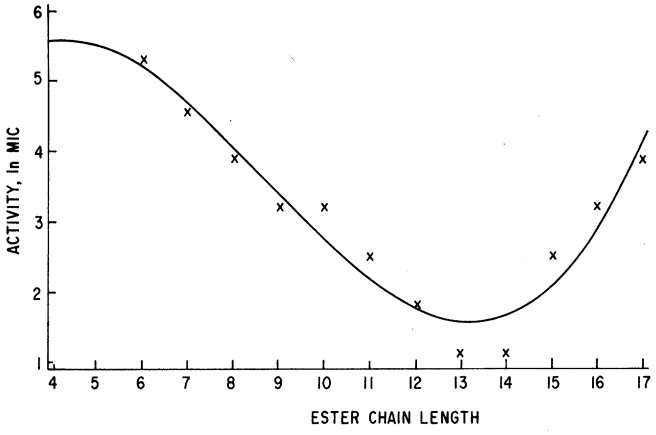

Figure 1. *Relationship of the chain length (R) of formic acid esters with the minimum inhibitory activity (MIC) against* C. botulinum. *Crosses indicate experimental data; the solid line indicates computerized approximation described by the cubic equation.*

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